Intracellular tumor-associated antigens represent effective targets for passive immunotherapy

Noguchi, T ; Kato, T ; Wang, L ; Maeda, Y ; Ikeda, H ; Sato, E ; Knuth, A ; Gnjatic, S ; Ritter, G ; Sakaguchi, S ; Old, L J ; Shiku, H ; Nishikawa, H

Abstract: Monoclonal antibody (mAb) therapy against tumor antigens expressed on the tumor surface is associated with clinical benefit. However, many tumor antigens are intracellular molecules that generally would not be considered suitable targets for mAb therapy. In this study, we provide evidence challenging this view through an investigation of the efficacy of mAb directed against NY-ESO-1, a widely expressed immunogen in human tumors that is expressed intracellularly rather than on the surface of cells. On their own, NY-ESO-1 mAb could neither augment antigen-specific CD8(+) T-cell induction nor cause tumor eradication. To facilitate mAb access to intracellular target molecules, we combined anti-NY-ESO-1 mAb with anticancer drugs to accentuate the release of intracellular NY-ESO-1 from dying tumor cells. Strikingly, combination therapy induced a strong antitumor effect that was accompanied by the development of NY-ESO-1-specific effector/memory CD8(+) T cells that were not elicited by single treatments alone. The combinatorial effect was also associated with upregulation of maturation markers on dendritic cells, consistent with the organization of an effective antitumor T-cell response. Administration of Fc-depleted F(ab) mAb or combination treatment in Fc receptor-deficient host mice abolished the therapeutic effect. Together, our findings show that intracellular tumor antigens can be captured by mAbs and engaged in an efficient induction of CD8(+) T-cell responses, greatly expanding the possible use of mAb for passive cancer immunotherapy.

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Takuro Noguchi, Takuma Kato, Linan Wang, et al.

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Takuro Noguchi[1, 4, 8], Takuma Kato[2], Linan Wang[1,3], Yuka Maeda[1,5], Hiroaki Ikeda[3], Eiichi Sato[6], Alexander Knuth[7], Sacha Gnjatic[5,8], Gerd Ritter[8], Shimon Sakaguchi[5], Lloyd J. Old[8], Hiroshi Shiku[1,3] and Hiroyoshi Nishikawa[1,5]

Departments of [1]Cancer Vaccine, [2]Cellular and Molecular Immunology, [3]Immuno-Gene Therapy, Mie University Graduate School of Medicine, Mie 514-8507, Japan, [4]Department of Surgical Oncology, Hokkaido University Graduate School of Medicine, Hokkaido 060-8638, Japan, [5]Experimental Immunology, Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan, [6]Department of Anatomic Pathology, Tokyo Medical University, Tokyo 160-8402, Japan, [7]Department of Oncology, University Hospital Zurich, Zurich 8091, Switzerland, [8]Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, New York, NY 10065.

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Correspondence should be addressed to:
Hiroyoshi Nishikawa, M.D., Ph.D.
Experimental Immunology, Immunology Frontier Research Center,
Osaka University
3-1 Yamadaoka, Suita, Osaka 565-0871, Japan.
Tel: +81-6-6879-4963 / Fax: +81-6-6879-4464
E-mail: nisihiro@ifrec.osaka-u.ac.jp

Hiroshi Shiku, M.D., Ph.D.
Departments of Cancer Vaccine and Immuno-Gene Therapy,
Mie University Graduate School of Medicine,
2-174 Edobashi, Tsu, Mie 514-8507, Japan.
Tel: +81-59-231-5062 / Fax: +81-59-231-5276
E-mail: shiku@clin.medic.mie-u.ac.jp

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Abbreviation: Cancer/testis antigen; CT antigen, draining lymph nodes; dLN, mutated MAP kinase, ERK2; mERK2, 5-fluorouracil; 5-FU, Immune complex; IC.

Abstract

Monoclonal antibody (mAb) therapy against tumor antigens expressed on the tumor surface is associated with clinical benefit. However, many tumor antigens are intracellular molecules that generally would not be considered suitable targets for mAb therapy. In this study, we provide evidence challenging this view through an investigation of the efficacy of mAb directed against NY-ESO-1, a widely expressed immunogen in human tumors that is expressed intracellularly rather than on the surface of cells. On their own, NY-ESO-1 mAb could neither augment antigen-specific $CD8^+$ T cell induction nor cause tumor eradication. To facilitate mAb access to intracellular target molecules, we combined anti-NY-ESO-1 mAb with anti-cancer drugs to accentuate the release of intracellular NY-ESO-1 from dying tumor cells. Strikingly, combination therapy induced a strong anti-tumor effect that was accompanied by the development of NY-ESO-1-specific effector/memory $CD8^+$ T cells that were not elicited by single treatments alone. The combinatorial effect was also associated with upregulation of maturation markers on dendritic cells, consistent with the organization of an effective antitumor T cell response. Administration of Fc-depleted Fab mAb or combination treatment in Fcγ receptor-deficient host mice abolished the therapeutic effect. Together, our findings demonstrate that intracellular tumor antigens can be captured by mAbs and engaged in an efficient induction of $CD8^+$ T cell responses, greatly expanding the possible use of mAb for passive cancer immunotherapy.

Introduction

With the molecular identification of tumor antigens recognized by the human immune system, antigen-specific immunotherapy for cancers has been developed and is explored in the clinic (1-3). Particularly, mAbs that recognize surface antigens, such as trastuzumab (anti-Her2/neu) and rituximab (anti-CD20), as a single agent or in combination with chemotherapy, are used in the clinic for frontline or salvage therapy and have resulted in objective and durable clinical responses (3-5). One of the major therapeutic mechanisms of mAb is considered to be the selective interruption of vital signaling pathways in which the targeted antigens are critically involved (3, 5). In addition, there is accumulating evidence that mAb therapy also works through Ab-dependent cellular cytotoxicity (ADCC) by NK cells or through the activation of complement, both of which depend on the Fc portion of the mAbs (6-9). Furthermore, Fc receptor-mediated uptake of immune complexes results in activation of APCs and facilitates cross-presentation of those antigens to tumor-specific $CD8^+$ T cells and inhibition of tumor growth, as was shown recently in HER2/*neu* and melanoma differentiation antigen tyrosinase-related protein-1 (Trp1; gp75) models (10-13).

However, many well-characterized tumor-associated antigens including cancer/testis (CT) antigens are intracellular antigens and thus not accessible for Abs (14-16). An exception is mAb TA99, which targets gp75 and was shown to induce NK and $CD4^+$ T cell dependent anti-tumor responses in vivo (17). However, the fact that gp75 is expressed both on the cell surface as well

as intracellularly makes it difficult to defining the precise targets for the anti-tumor responses induced by mAb TA99 (12, 17).

NY-ESO-1, a CT antigen discovered by SEREX (serological identification of antigens by recombinant expression cloning) using the serum of an esophageal cancer patient, is frequently expressed in cancer cells of various tissue origins, but not in normal somatic cells except for germ cells in the testis (2, 18). Spontaneous cellular and humoral immune responses against NY-ESO-1 are found in cancer patients, which underscores its immunogenicity (2, 18). It has an intracellular location and lacks cell surface expression (2, 18), thus curtails it from being a candidate of mAb therapy. Interestingly, NY-ESO-1 protein/ IgG Ab complexes (immune complexes: IC) are efficiently cross-presented to the MHC-class I pathway (19, 20) and there is a close correlation between Ab and $CD8^+$ T cell responses (2, 21), suggesting that NY-ESO-1-specific $CD8^+$ T cell induction by cross-priming *in vivo* is associated with the induction of specific Abs. These data prompted us to analyze the possibility whether mAb therapy could be applied to an intracellular molecule NY-ESO-1 and inhibit tumor growth by enhancing $CD8^+$ T cell induction.

We have established syngeneic tumor models in BALB/c mice using CT26 colon carcinoma cells and CMS5a sarcoma cells that are stably transfected with NY-ESO-1 (22, 23). Using these models, we addressed whether NY-ESO-1 mAb combined with chemotherapy augmented NY-ESO-1-specific $CD8^+$ T cell induction and inhibited tumor growth.

Materials and Methods

Mice

Female BALB/c mice and BALB/c$^{nu/nu}$ mice were obtained from SLC Japan or Jackson laboratory and used at 7-10 weeks of age. BALB/c mice deficient in the γ chain subunit of Fc receptors were obtained from Taconic and used at 7-10 weeks of age. Mice were maintained in accordance with the NIH and American Association of Laboratory Animal Care regulations. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine and by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

Tumors

CT26 is a colon epithelial tumor derived by intrarectal injections of N-nitroso-N-methylurethane in BALB/c mice (24). CT26 expressing NY-ESO-1 (CT26-NY-ESO-1) was established as described (2, 22, 23). CMS5a is a subcloned cell line obtained from CMS5 (25). CMS5a-NY-ESO-1 was established as described (22).

Antibodies and reagents

Anti-NY-ESO-1 mAbs [E978 (mouse IgG1) recognizing NY-ESO-1$_{71-90}$, ES121 (mosue IgG1) recognizing NY-ESO-1$_{91-110}$, 219-510 (mouse IgG1) recognizing NY-ESO-1$_{21-40}$, [Supplemental Figure 3, (26)], anti-CD4 (GK1.5, rat IgG2b), anti-CD8 (19/178, mouse IgG2a) and anti-MAGE-A4 (MCV1, mouse IgG1)

were purified from hybridoma supernatant by protein G affinity chromatography. The Fab fragment of E978 was generated using the ImmunoPure Fab Preparation Kit (Thermo Fisher Scientific). Anti-CD8 (53-6.7), anti-CD45RB (16A), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-CD40 (3/23), anti-IFN-γ (XMG1.2), anti-CD62L (MEL-14), anti-CD11c (HL3), anti-TNF-α (MP6-XT22) and anti-mouse IgG1 (A85-1) mAbs were purchased from BD Biosciences, Biolegend or eBioscience. PE-labeled NY-ESO-1_{81-88}-D^d tetramers were provided from Drs. P. Guillaume and I. Luescher (Ludwig Institute Core Facility, Lausanne, Switzerland). An anti-NY-ESO-1 human IgG1 mAb (12D7) was obtained from CT Atlantic (Schlieren, Switzerland). p63 (T) peptide TYLPTNASL (27), AH-$1_{138-147}$ peptide SPSYVHQF (28) and NY-ESO-1_{81-88} peptide RGPESRLL (23) were purchased from Operon Biotechnologies (Tokyo, Japan), BioSynthesis (Lewisville, TX) and Sigma.

Chemotherapeutic agents

5-fluorouracil (5-FU, Kyowa Hakko Kirin, Tokyo, Japan), doxorubicin (Kyowa Hakko Kirin), CPT-11 (Yakult, Tokyo, Japan), paclitaxel (Bristol-Myers Squibb), cyclophosphamide (Shionogi, Osaka, Japan) were injected intraperitoneally as indicated.

Tumor challenge

Mice were inoculated with 0.5 - 1 x 10^6 CT26-NY-ESO-1 cells, 1 x 10^6 CMS5a-NY-ESO-1, or 1 x 10^6 CT26-MAGE-A4 cells in the right hind flank subcutaneously. Mice were monitored three times a week and were sacrificed when tumors reached >20 mm.

Staining and flow cytometry

To collect tumor infiltrating T cells, tumors were minced and treated with 1 mg/mL of collagenase IA (Sigma) in HBSS for 90 min at room temperature.

Cells harvested from draining lymph node (dLN) and tumors were stained for surface markers in PBS with 0.5% FBS for 15 min at 4ºC. For intracellular cytokine staining, 1-3 x 10^6 cells from tumors or dLN were cultured with peptide for 5h at 37 ºC, and GolgiPlug was added for the last 4h of culture. These cells were stained for surface markers and intracellularly with APC-conjugated anti-IFN-γ and PE-conjugated anti-TNF-α mAbs after permiabilization and fixation using Cytofix/Cytoperm Kit (BD Bioscience). Dead cells were excluded by LIVE/DEAD Fixable Dead Cell Stain Kits (Invitrogen). Cells were analyzed on FACSCanto or FACScalibur (BD Bioscience) and FlowJo software (Tree Star, Ashland, OR).

Fluorescent immunohistochemistry

Three μm tissue sections prepared from fresh frozen tumor specimens were fixed with ice-chilled acetone for 15 minutes. Alexa 488-labeled anti-human IgG Ab (Invitrogen) was applied and incubated at room temperature for 2 hrs. For double immunolabeling, sections were fixed with 3% paraformaldehyde for 15 minutes, incubated with anti-cleaved caspase 3 (Cell signaling technology, Beverly, MA) at room temperature for 2hrs, and then incubated Alexa-488-labeled anti-human IgG antibody and Alexa-568-labeled anti-rabbit IgG Ab (Invitrogen) at room temperature for 2hrs. Sections were rinsed with PBS, counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted.

Images were captured using x40 magnification objective by Zeiss AxioCam system (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Tumor curves were assessed by one-way ANOVA with a Bonferroni multiple comparisons posttest. Single measurement comparison between two groups was evaluated by two-sided Student's *t* test. P values < 0.05 were considered statistically significant.

Results

Establishment of CT26-NY-ESO-1

We established a syngeneic colon carcinoma model (CT26-NY-ESO-1) with stable NY-ESO-1 expression (2, 22, 23). NY-ESO-1 expression in CT26-NY-ESO-1 cells was exclusively intracellular, and no NY-ESO-1 protein was detected on the cell surface (Supplemental Figure 1A), consistent with the expression of NY-ESO-1 protein in human cancer cells (2). These CT26-NY-ESO-1 cells maintained the same tumor growth capacity as their parental CT26 cells in both wild-type Balb/c and C.B-17 Scid mice, indicating that there was no alteration of tumorigenicity caused by the NY-ESO-1 transfection (Supplemental Figure 1B). When BALB/c mice were inoculated with CT-26-NY-ESO-1 cells, spontaneous Ab and $CD8^+$ T cell responses were detected after 7 days and increased thereafter (Supplemental Figure 1C and 1D). These spontaneous immune responses closely paralleled spontaneous NY-ESO-1-specific immune responses found in humans (2).

We used this tumor model to explore the anti-tumor effects of mAbs against NY-ESO-1 alone and in combination with an anti-cancer drug. To select anti-cancer drugs suitable for this model, we examined the anti-tumor capacity of several anti-cancer drugs (5-FU, CPT-11, Paclitaxel, and Doxorubicin) against CT26-NY-ESO-1. Of the four drugs, 5-FU exhibited a significant anti-tumor effect (Supplemental Figure 2A). When CT-26-NY-ESO-1 cells were cultured with 5-FU, NY-ESO-1 protein was released from CT-26-NY-ESO-1 cells into the culture supernatant, but not from

parental CT26 cells, (Supplemental Figure 2B). Based on these data, we chose 5-FU for our further experiments.

Combination treatment with anti-NY-ESO-1 mAb and 5-FU results in augmented tumor growth inhibition.

BALB/c mice were inoculated with CT26-NY-ESO-1 and were injected with 5-FU (75 mg/kg) and anti-NY-ESO-1 mAb (clone; E978, 100 μg, two days after 5-FU injection) when the tumor was palpable (around 25 mm^2). Treatment was repeated after one week. The combination treatment with anti-NY-ESO-1 mAb and 5-FU exhibited a significantly augmented anti-tumor effect and longer survival compared with control mice or mice that had received either 5-FU or anti-NY-ESO-1 mAb alone (Figure 1A and 1B). This augmented anti-tumor effect was also observed when another anti-NY-ESO-1 mAb (clone; ES121, 100 μg) was used, but not with a control mAb, against another immunogenic cancer/testis antigen MAGE-A4, which is not expressed in the CT26-NY-ESO-1 cells (Figure 1C and 1D). In contrast, combination treatment with anti-MAGE-A4 mAb (clone; MCV1, 100 μg), but not control Ab and 5-FU exhibited an augmented anti-tumor effect against CT-26-MAGE-A4 (Figure 1E). To show the effect of this combination treatment is not limited to the CT26, we examined the anti-tumor effect using CMS5a fibrosarcoma cells. BALB/c mice were inoculated with CMS5a-NY-ESO-1 and were injected with doxorubicin (50 μl intratumoral injection, 0.25 mM) and anti-NY-ESO-1 mAb. As systemic administration of doxorubicin did not induce effective killing of CMS5a-NY-ESO-1, we employed an intratumoral injection method. This combination treatment with anti-NY-ESO-1 mAb (but not an isotype control Ab)

and doxorubicin exhibited a significantly augmented anti-tumor effect as well (Figure 1F). These data suggest that the augmented anti-tumor effect is an antigen-specific phenomenon and that this combination treatment could be applicable to a broader range of intracellular antigens and tumors.

We next investigated whether a cocktail of two different anti-NY-ESO-1 mAbs (E978 50 μg and ES121 50 μg) that recognize two different non-overlapping epitopes on the NY-ESO-1 protein (Supplemental Figure 3) further augmented anti-tumor effects. We observed no additive anti-tumor effects when mice were treated with the combination of two different anti-NY-ESO-1 mAbs and 5-FU compared with mice treated with a single anti-NY-ESO-1 mAb and 5-FU (Figure 1G).

Augmented tumor growth inhibition by combination treatment with anti-NY-ESO-1 mAb and 5-FU is dependent on $CD8^+$ T cells.

To gain insight into the cellular components involved in the augmented anti-tumor effects by the combination treatment, we initially examined the role of T cells using BALB/$c^{nu/nu}$ mice. BALB/$c^{nu/nu}$ mice were inoculated with CT26-NY-ESO-1 and combination treatment with 5-FU and anti-NY-ESO-1 mAb was initiated when the tumor was palpable. The augmented anti-tumor effect by the combination treatment in wild-type BALB/c mice was abrogated in BALB/$c^{nu/nu}$ mice (Figure 2A).

Given the critical role of T cells in this augmentation of anti-tumor effects, we next explored the outcome of $CD4^+$ / $CD8^+$ T cell depletion on the augmented anti-tumor effect. BALB/c mice bearing CT26-NY-ESO-1 tumors were injected with 5-FU and anti-NY-ESO-1 mAb and received anti-CD4 (day

7, 14 and 21) or anti-CD8 mAb (day 7 and 21). The depletion of $CD8^+$ T cells totally abolished the augmented anti-tumor effects (Figure 2B). In contrast, $CD4^+$ T cell depletion did not affect the augmented anti-tumor effects (Figure 2B).

Combination treatment with anti-NY-ESO-1 mAb and 5-FU enhances NY-ESO-1-specific $CD8^+$ T cell induction.

Considering a critical role of $CD8^+$ T cells, we examined NY-ESO-1-specific T cells in dLN. BALB/c mice were inoculated with CT26-NY-ESO-1 and received the combination treatment. dLN and tumors were harvested on day 14-16 and cells were incubated with NY-ESO-1_{81-88} (23) or control peptide, and cytokine secretion was analyzed. Combination treatment with anti-NY-ESO-1 mAb and 5-FU elicited significantly higher numbers of NY-ESO-1-specific $CD8^+$ T cells producing IFN-γ and/or TNF-α compared with 5-FU alone (Figure 3A). Further, there was a trend to higher numbers of NY-ESO-1-specific $CD8^+$ T cells in tumors treated with the combination treatment compared with those treated with 5-FU alone (Figure 3C).

To explore further differences in NY-ESO-1_{81-88}-specific $CD8^+$ T cells, the effector/memory status was analyzed. The frequency of NY-ESO-1-specific $CD8^+$ T cells as measured by $CD8^+$NY-ESO-1/D^d $tetramer^+$ T cells was higher in mice treated with the combination therapy as compared to mice treated with 5-FU alone, confirming the data from the intracellular cytokine assays. The frequency of effector/memory ($CD62L^{low}CD45RB^{low}$) T cells was higher in mice treated with the combination treatment (Figure 3B). In contrast, frequency of naive ($CD62L^{high}CD45RB^{high}$) T cells was higher in mice

treated with 5-FU alone, indicating that the combination treatment efficiently activated antigen-specific $CD8^+$ T cells.

Therapeutically effective antigen spreading is observed in mice treated with the combination treatment.

Certain immunization strategies result in the development of an immune response against tumor antigens that are not contained in the vaccine but are found in tumor cells, a phenomenon known as antigen spreading (22, 29, 30). Therefore, we explored whether the combination treatment resulted in the development of an immune response against other antigens expressed in tumor cells. As we employed CT26 tumors, we examined $CD8^+$ T cells recognizing AH-1 peptide, which is derived from the envelope protein (gp70) of an endogenous ecotropic murine leukemia provirus expressed by CT26 and previously shown to be a target of $CD8^+$ T cells (28). Mice bearing CT26-NY-ESO-1 received treatment with anti-NY-ESO-1 mAb and 5-FU. Given that antigen spreading is observed after the antigen release from killed tumor cells, AH-1-specific $CD8^+$ T cell induction was analyzed at later time point (day 24). Significantly higher numbers of AH-1-specific $CD8^+$ T cells was detected in mice treated with anti-NY-ESO-1 mAb and 5-FU compared with mice treated with 5-FU alone (Figure 4).

The Ab Fc portion is required for the augmented anti-tumor effect by the combination treatment.

We next explored the mechanism(s) of the augmented anti-tumor effect and the differences of NY-ESO-1_{81-88}-specific $CD8^+$ T cells. MAb therapy can

exhibit immunostimulatory effects through the Fc portion of a mAb (7, 8). We investigated whether the augmented anti-tumor effect by combination treatment depended on the Fc portion of the mAb. BALB/c mice bearing CT26-NY-ESO-1 tumors were injected with 5-FU (day 7 and 14) and intact Ab or an Fc-depleted form of the anti-NY-ESO-1 mAb (day 9 and 16). The anti-tumor effect induced by the combination treatment with the intact anti-NY-ESO-1 mAb and 5-FU was totally abolished when Fab antibodies were administered (Figure 5A). We further examined the critical role of the Fc portion for this augmented anti-tumor effect by the combination treatment using activating Fcγ receptor knockout mice (Fcer1g$^{-/-}$ mice). In these mice, we did not observe the augmented anti-tumor effect by the combination treatment compared with mice treated with 5-FU alone (Figure 5B), confirming the critical role of the Ab Fc portion for this augmented anti-tumor effect.

Accumulation of Ab to tumor sites by combination treatment

Given the importance of the Fc portion and the antigen-Ab IC formation for an enhancement of CD8$^+$ T cells (19-21), we examined the accumulation of the anti-NY-ESO-1 mAb to tumor sites for assessing the *in vivo* formation of antigen-Ab IC. To this purpose, we utilized a human anti-NY-ESO-1 mAb to detect and visualize the accumulation of anti-NY-ESO-1 mAb at the tumor sites. BALB/c mice bearing CT26-NY-ESO-1 received 5-FU and human anti-NY-ESO-1 mAb two days later. Tumors were removed several time points after the mAb injection. Anti-NY-ESO-1 mAb accumulated in CT26-NY-ESO-1 tumors after 24 hrs and maintained thereafter when given in combination with 5-FU (Figure 6A and 6B). In contrast, the accumulation of anti-NY-ESO-1 mAb

in the tumors was lower without 5-FU treatment (Figure 6A and 6B). We next tested whether the released NY-ESO-1 protein localized around the area of 5-FU-induced cell death. Anti-NY-ESO-1 mAb accumulated around the apoptotic area detected by cleaved caspase 3 staining (Figure 6B), suggesting that 5-FU accentuated the natural release of intracellular NY-ESO-1 from dying tumor cells subsequently resulting in an increased accumulation of anti-NY-ESO-1 mAb in tumors and the formation of antigen-Ab IC.

Formation of antigen-Ab IC in-vivo by the combination treatment induces sufficient maturation of DCs for tumor eradication.

We next analyzed the role of DCs for this augmentation of anti-tumor effects. The activation status (CD80, CD86, MHC class II and CD40) of $CD11c^+$ DCs at dLN after treatment was examined. The expression level of CD80, CD86, MHC class II and CD40 in DCs was significantly enhanced in mice that received the combination treatment with anti-NY-ESO-1 mAb and 5-FU compared to mice treated with 5-FU alone (Figure 6C).

Discussion

In view of the recent clinical successes of targeted mAbs to tumor antigens expressed on the surface of tumors for cancer therapy (3-5), we explored the feasibility to extend this approach of targeted mAb therapy to intracellular molecules as the majority of tumor antigens identified to date, are exclusively expressed and located inside the cell (14-16). Appropriate maneuvers that facilitate access of mAbs to these intracellular antigenic targets are critical requirement for this approach. Nucleoside analogues, such as 5-FU, predominantly induce apoptosis in target cells (31), but we found that NY-ESO-1 protein was released from tumor cells after 5-FU treatment in similar amounts as released by necrosis. The injected mAb accumulated into CT26-NY-ESO-1 tumors, suggesting the *in vivo* formation of antigen-Ab ICs. Furthermore, DCs in dLN that captured these ICs exhibited a mature phenotype and were associated with the induction of higher numbers of NY-ESO-1-specific $CD8^+$ T cells. This augmented anti-tumor immunity by combination treatment with anti-NY-ESO-1 mAb and 5-FU was abrogated in nude mice and by deleting $CD8^+$ cells, arguing that a major involvement of Ab-dependent cellular cytotoxicity (ADCC) or complement is less likely. Furthermore, this augmented anti-tumor effect by intracellular antigen-specific mAb combined with chemotherapy was observed in another tumor system using doxorubicin, indicating the broader application of this combination treatment.

A combination of anti-Her2 mAb and HER2/neu-expressing GM-CSF-secreting tumor vaccine augmented the anti-tumor effect compared

with either treatment alone, and the improved therapeutic efficacy depended on Fc-mediated activation of APCs (11). TA-99 (recognizing Trp1) mAb enhanced DNA vaccination-induced anti-tumor effects (12). More recently, Park et al. showed that the therapeutic effect of an anti-HER2/neu mAb was associated with adaptive cellular immune responses, such as $CD8^+$ T cells (13). While these data clearly implicated a critical role for Fc-mediated APC activation and cross-priming correlated with enhancement of antigen-specific $CD8^+$ T cell induction, other or additional mechanisms may include direct signal blocking and other Fc-mediated anti-tumor effects as the target antigens were expressed on the cell surface. These data, therefore, do not unambiguously suggest a possible application of mAb therapy to intracellular molecules. Here, we show that Fc-mediated antigen-specific $CD8^+$ T cell induction was an important element of mAb therapy using mAbs against tumor antigens that are exclusively expressed in the intracellular compartment and we suggest the potential application of targeted mAb therapy also to intracellular tumor antigens. As a result, it is of interest to re-address the correlation between anti-tumor effect of $CD8^+$ T cell response and clinical response by Trastuzumab (anti-Her2/neu) treatment, as Trastzumab is able to enhance cross-presentation *in vitro* (32).

Another unique point in our study is that our mAb treatment targeting an intracellular antigen does not require *in vitro* formation of IC or a combination with antigen immunization, such as protein or DNA vaccines for the formation of antigen-Ab IC (10-12, 33). When the mAb was injected alone, an augmented anti-tumor effect was not observed in our model, suggesting the essential role of chemotherapy for releasing sufficient amounts of antigen to

form antigen-Ab IC. Other modalities for facilitating antigen release from tumors such as radiation therapy, cryo-ablation or other agents that may result in partial destruction of tumor cells could be applicable to this combination therapy. These results are particularly important for considering the clinical application of targeted mAb therapy because combinations of chemotherapy and mAbs have already been widely used in the clinic (3-5). Furthermore, combining a mAb therapy with protein or DNA cancer vaccines is very expensive and enormous effort is required to translate into the clinic.

$CD4^+$ T cell help is necessary for a proper activation and a long-lasting memory formation of $CD8^+$ T cells (34, 35). While combination treatment with anti-NY-ESO-1 mAb and chemotherapy provided an augmented anti-tumor efficacy and induced higher numbers of NY-ESO-1-specific $CD8^+$ T cells with effector-memory type, these effects were dependent on $CD8^+$ T cells, but not $CD4^+$ T cells. One can envisage that since a major role of $CD4^+$ T cells is to stimulate APCs, such as DCs to activate $CD8^+$ T cells (licensing) (34, 35), signals provided through Fc receptors may compensate the $CD4^+$ T cell help for stimulating/activating APCs. Alternatively, inflammation induced by anti-cancer drugs further supports the stimulating/activating of APCs.

One intriguing question is why the combination of mAb and 5-FU exhibited a strong anti-tumor effect, despite a possible inhibitory signal through a subclass of IgG, namely IgG1 used in this study (8). Since we employed anti-NY-ESO-1 mAbs (mouse IgG1) for this combination therapy, IgG1 may show inhibitory function by activating inhibitory Fc receptor (7-9). Some protocols of anti-cancer chemotherapy induce the stimulation of immune

responses by Toll-like receptor ligands released from tumor cells (36). The possibility that 5-FU-induced tumor destruction stimulates inflammation signals, such as Toll-like receptor signals, and these inflammation signals may change the ratio of stimulatory/inhibitory Fc receptor expression to a more stimulatory condition (8) is less likely, because our preliminary data shows that the balance between activating Fcγ receptor III and inhibitory Fcγ receptor IIB expression on $CD11c^+$ cells was not influenced by 5-FU treatment. This raises several possibilities; 1) The balance between those receptors changes on other hematopoietic cells, 2). Signaling pathways through those Fcγ receptors are altered by chemotherapy-induced inflammation, 3). Ab specificity is not good enough to address this point and proper knockout animals are required. In addition, it will be crucial to compare the effect of immunological responses by other IgG subclasses, and studies with class-switched Abs and with Fcγ receptor IIB knockout mice are planned.

We observed that mAb and 5-FU combination treatment resulted in the development of an immune response against tumor antigens that have not been directly targeted by the Ab but that are expressed in tumor cells, a phenomenon known as antigen spreading (22, 29, 30). While mice treated with 5-FU alone or without treatment elicited NY-ESO-1-specific $CD8^+$ T cell responses, antigen spreading and its therapeutic effectiveness were limited in these mice. It is also possible that efficient activation of DCs by the targeted mAb and 5-FU combination treatment provides the opportunity to stimulate subsequently additional $CD8^+$ T cells specific for other antigens derived from the tumor cells. Therefore, effective anti-tumor responses, such as tumor eradication, may require $CD8^+$ T cells specific for the single antigen used for

immunization, but also multiple antigens that were contained in tumors, as shown in other murine systems and human cancer vaccines (1, 22, 37, 38).

In our model as well as in cancer patients, NY-ESO-1 humoral responses could be spontaneously elicited. While a correlation between humoral responses and longer survival was not reported, NY-ESO-1-specific $CD8^+$ T cell induction by cross-priming *in vivo* is associated with the induction of specific Abs (2, 39). Spontaneous NY-ESO-1 humoral responses are correlated with progression of tumor stage in humans (2, 39). In our mouse system, spontaneously-induced anti-NY-ESO-1 Abs were observed when tumors reached a larger size. The level of spontaneously-induced Abs is about 10-times lower than that achieved by mAb injection (Supplemental Figure 1C), suggesting that spontaneously-induced humoral responses may potentially have some anti-tumor effects, but the amount of Abs may be too low to exhibit effective anti-tumor activity, such as facilitating tumor regression. Our data revealed that mAb and 5-FU combination treatment induced higher numbers of effector/memory NY-ESO-1-specific $CD8^+$ T cells compared with chemotherapy alone, reflecting a long lasting anti-tumor capacity as shown by improved survival. In conclusion, combination treatment with targeted mAbs and chemotherapy opens a new era of Ab cancer immunotherapy for tumor antigens with intracellular expression.

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Figure Legends

Figure 1. Combination of mAb and an anti-cancer drug exhibits augmented tumor growth inhibition.

(A-D, G) BALB/c mice were inoculated with CT26-NY-ESO-1 and treatment was started when tumors were palpable (around 25 mm^2, day 5-7). Mice received 5-FU intravenously (black arrow) and/or anti-NY-ESO-1 mAb (clone; E978 or ES121) or anti-MAGE-A4 mAb (clone; MCV1) two days after 5-FU injection (red arrow). Treatment was repeated twice at one-week intervals. (A) Left panel: tumor growth curves representative of two independent experiments, right panel: summary of tumor size on day 24 of two independent experiments. (B) Survival curves summarized from another 3 independent experiments (separate from tumor growth data) are shown. (C, D) Left panels: tumor growth curves representative of two independent experiments, right panels: summary of tumor size of two independent experiments on day 23 and on day 21, respectively. (E) BALB/c mice were inoculated with CT26-MAGE-A4 and treatment was started as in (A). Mice received 5-FU (black arrow) and anti-MAGE-A4 mAb (clone; MCV1) or anti-NY-ESO-1 mAb (clone; E978) two days after 5-FU injection (red arrow). Left panel: tumor growth curves representative of two independent experiments, right panel: summary of tumor size on day 23 of two independent experiments. (F) BALB/c mice were inoculated with CMS5a-NY-ESO-1 and treatment was started as in (A). Mice received doxorubicin (50 μl, 0.25mM) intratumorally (black arrow) and/or anti-NY-ESO-1 mAb (clone; E978) two and four days after doxorubicin administration (red arrow). Left panel: tumor growth curves representative of

two independent experiments, right panel: summary of tumor size on day 21 of two independent experiments. (G) Mice were injected with 5-FU (black arrow) and anti-NY-ESO-1 mAb (E978, 100 μg) or cocktail of two anti-NY-ESO-1 mAbs (E978 50 μg and ES121 50 μg) two days after 5-FU injection (red arrow). Left panel: tumor growth curves representative of two independent experiments, right panel: summary of tumor size on day 24 of two independent experiments. Tumor size was monitored three times a week. Each group consisted of 3-8 mice. Data are mean ±SD. N.S.: not significant.

Figure 2. Augmented tumor growth inhibition by the combination treatment depends on $CD8^+$ T cells.

(A) BALB/$c^{nu/nu}$ mice were inoculated with CT26-NY-ESO-1 and treatment with 5-FU (day 5 and 12, black arrow) and anti-NY-ESO-1 mAb (E978, day 7 and 14, red arrow) was started as in Figure 1A. Left panel: tumor growth curves representative of two independent experiments, right panel: summary of tumor size on day 19 of two independent experiments. (B) BALB/c mice bearing CT26-NY-ESO-1 were injected with 5-FU (day 7 and 14, black arrow) and anti-NY-ESO-1 mAb (E978, day 9 and 16, red arrow), and received anti-CD4 (day 7, 14 and 21, brown arrow) or anti-CD8 mAb (day 7 and 21, purple arrow), resulting in > 95% depletion of CD4/CD8 cell depletion. Left panel: tumor growth curves representative of two independent experiments, right panel: summary of tumor size on day 23 of two independent experiments. Tumor size was monitored three times a week. Each group consisted of 5-7 mice. Data are mean ±SD. N.S.: not significant.

Figure 3. Combination treatment enhances NY-ESO-1-specific $CD8^+$ T cell induction.

(A B, and C) BALB/c mice (n=3) were inoculated with CT26-NY-ESO-1 and injected with 5-FU and anti-NY-ESO-1 mAb (E978). (A) On day 14, dLN were removed and incubated with NY-ESO-1_{81-88} or control peptide. IFN-γ and TNF-α secretion by $CD8^+$ T cells was analyzed. (B) dLN cells were isolated on day 16, and CD45RB and CD62L expression on NY-ESO-1_{81-88}-specific $CD8^+$ T cells identified as $CD8^+$NY-ESO-1_{81-88}/D^d $tetramer^+$ T cells was analyzed. (C) Tumor infiltrating lymphocytes were collected on day 16, and incubated with NY-ESO-1_{81-88} or control peptide. IFN-γ and TNF-α secretion by $CD8^+$ T cells was analyzed. These experiments were repeated twice with similar results. Data are mean ±SD. EM: effector/memory T cells.

Figure 4. Antigen spreading is observed in mice that received the combination treatment.

BALB/c mice (n=3) were inoculated with CT26-NY-ESO-1 and injected with 5-FU and anti-NY-ESO-1 mAb (E978). On day 24, dLN were removed and incubated with AH-$1_{138-147}$ or control peptide. IFN-γ and TNF-α secretion by $CD8^+$ T cells was analyzed. These experiments were repeated twice with similar results. Data are mean ±SD.

Figure 5. The Fc receptor signals are required for augmented anti-tumor effects by the combination treatment.

(A) BALB/c mice were inoculated with CT26-NY-ESO-1 and injected with 5-FU (day 7 and 14) and intact or Fc-depleted Fab anti-NY-ESO-1 mAb (E978, day

9 and 16). (B) Fcγ receptor knockout mice were inoculated with CT26-NY-ESO-1 and injected with 5-FU (day 7 and 14) and anti-NY-ESO-1 mAb (E978, day 9 and 16). Tumor size was monitored three times a week. Each group consisted of 3-10 mice. Left panels: tumor growth curves representative of two independent experiments, right panels: summary of tumor size of two independent experiments on day 22 (A) and day 23(B). Data are mean ±SD. N.S.: not significant.

Figure 6. The combination treatment results in accumulation of injected antibody at the tumor site and induces maturation of DCs.

(A) BALB/c mice were inoculated with CT26-NY-ESO-1 and injected with 5-FU (day 5) and human anti-NY-ESO-1 mAb (12D7, day 7) or human anti-A33 mAb as a control (day 7). 24 hrs after mAb injection, tumors were removed and the accumulation of human anti-NY-ESO-1 mAb into tumors was examined by immunohistochemistry. (bar=50 μm) (B) CT26-NY-ESO-1 tumors as in (A) were removed at the indicated time points after mAb injection and co-stained with anti-human IgG mAb (green) and anti-cleaved caspase 3 mAb (red). (bar=50 μm) (C) Two days after the last 5-FU injection, dLN were harvested. CD80, CD86, CD40 and MHC class II expression on $CD11c^{high}$DCs was analyzed. These experiments were repeated twice with similar results.

Figure 1

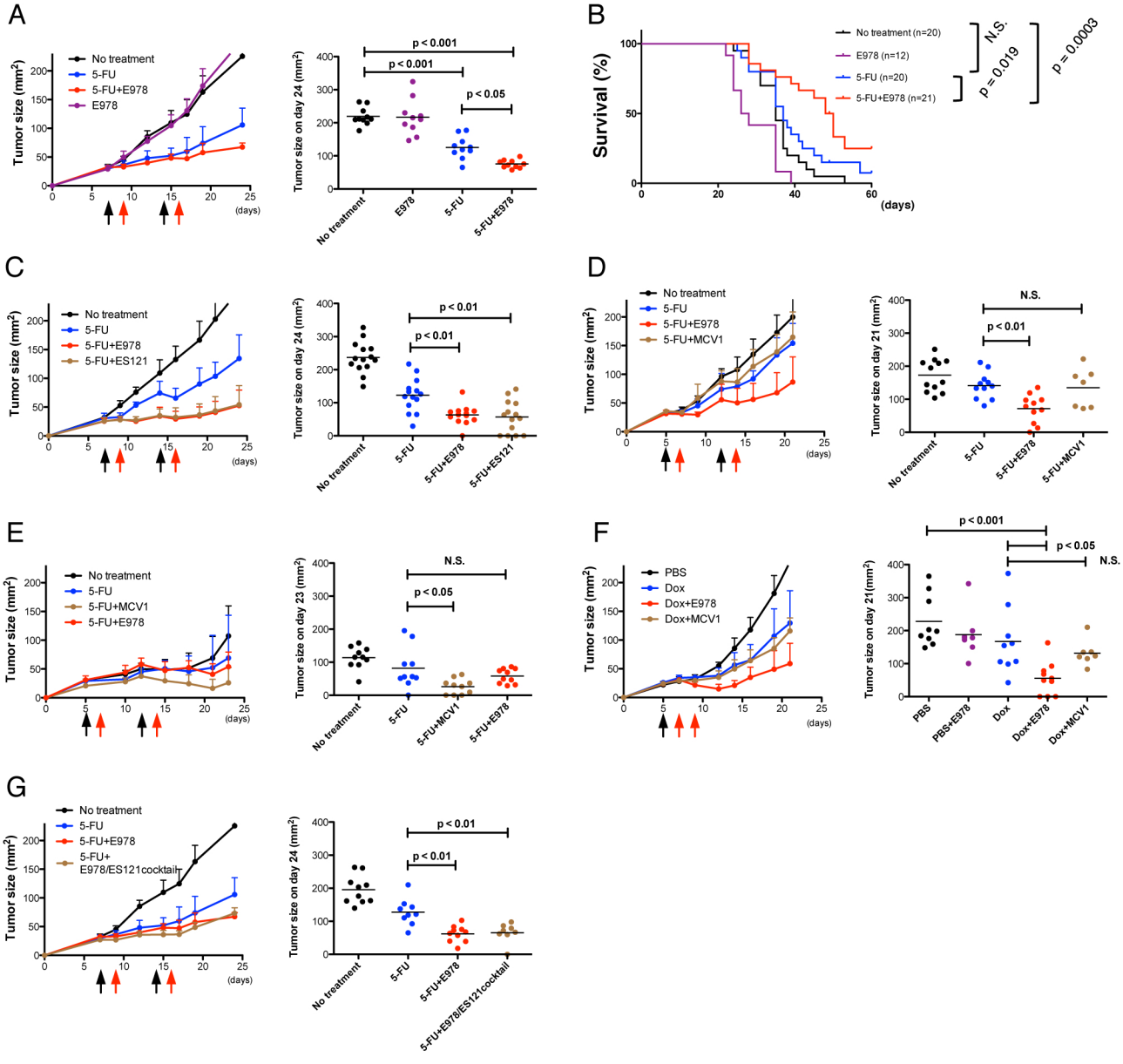
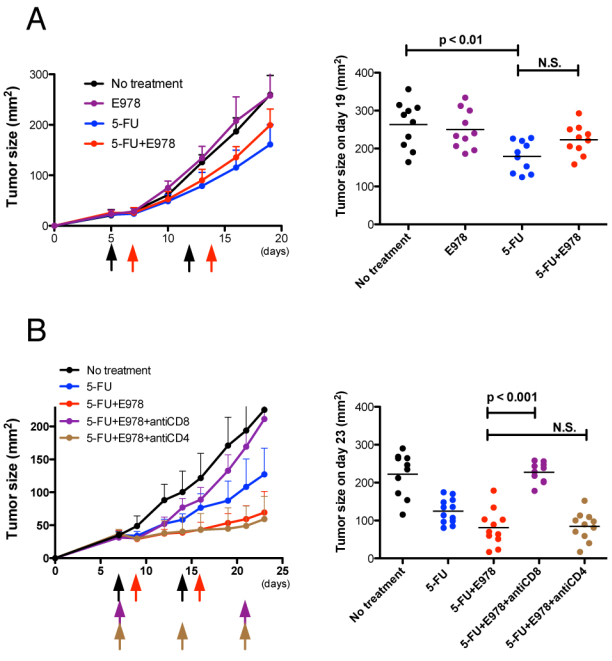


Figure 2



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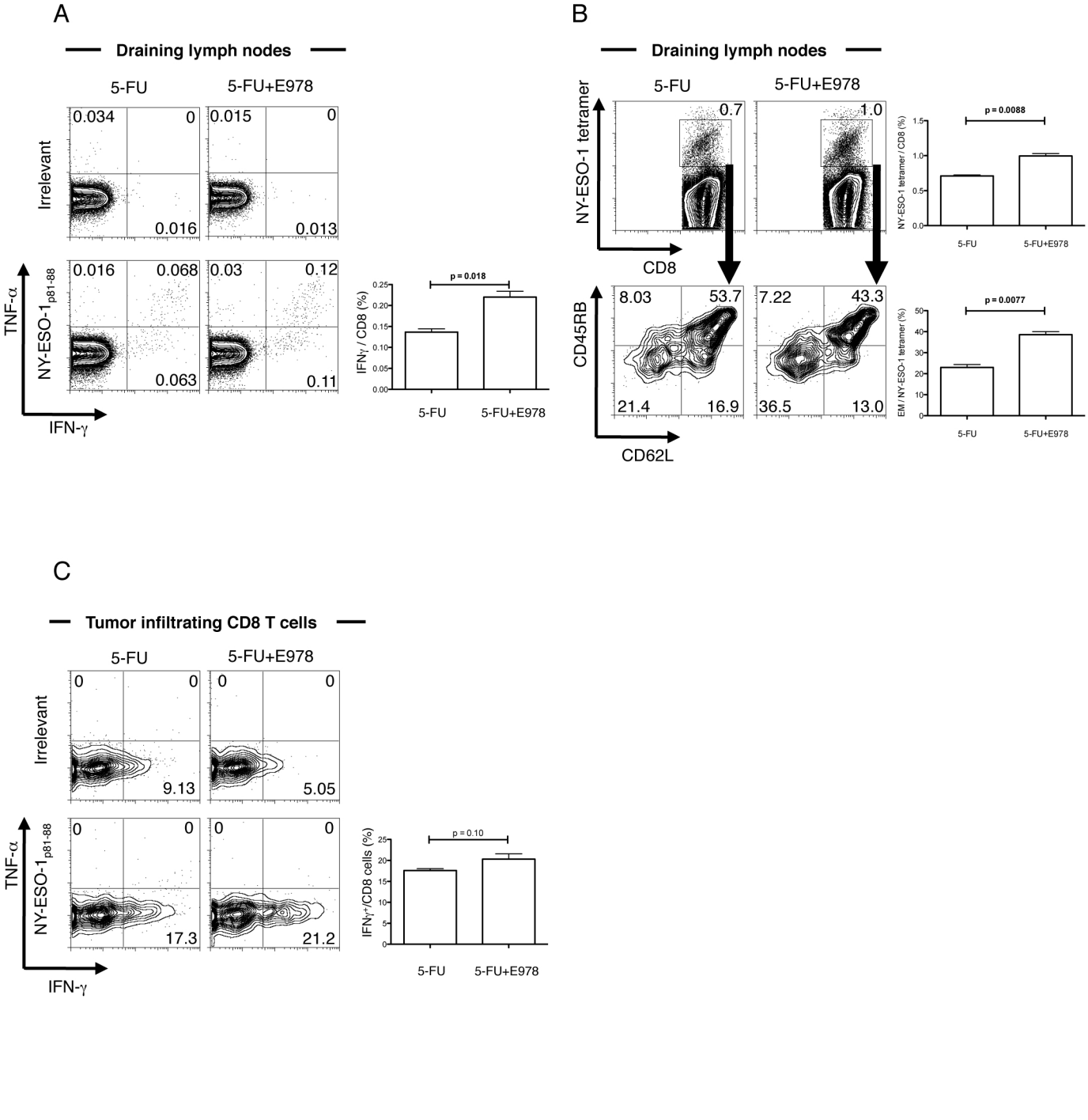


Figure 4

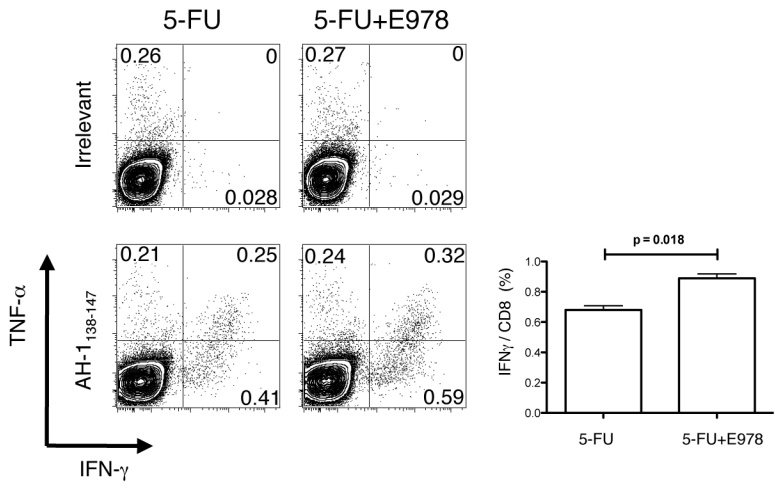
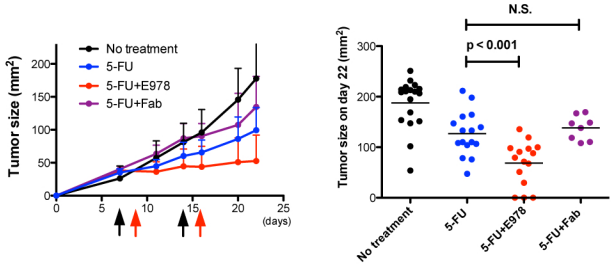


Figure 5

A



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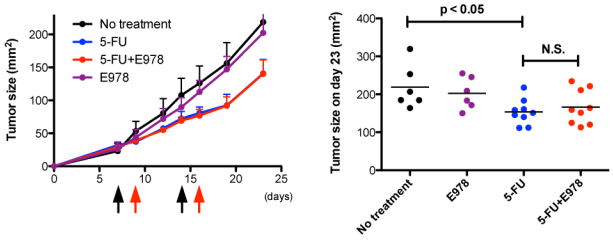

Figure 6